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# MOLECULAR DIAGNOSIS OF *CRYPTOSPORIDIUM PARVUM* IN DIARRHEAL PATIENTS OF WASSIT PROVINCE

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## ABSTRACT

Cryptosporidiosis is a disease caused by *Cryptosporidium* which is zoonotic protozoan parasite of the Apicomplexa phylum and the Cryptosporidiidae family. It is one of the most important pathogen in humans. The present study included the patients who were referred to Al Karamah Teaching Hospital and General Hospital of Martyr Fairuz at Wasit, Iraq during November 2015 to February 2016. A total of 100 children, aged from less than 1year to 11 years old, who suffered from watery diarrhea and abdominal pain were recruited. Stool samples were collected and examined using modified Zeihl-Neelson and Real-time PCR methods. The results showed that 29% of the samples were positive for *C. parvum* oocysts using modified Zeihl-Neelson and 26% of the samples were positive by Real-time PCR and 7 samples out of 71 samples negative in modified Zeihl-Neelson (7%), gave positive results by RT-PCR. The results showed that 33 samples out of 100 samples were gave positive by RT-PCR.

**KEYWORDS:** *C.parvum*, Diagnosis, Modified Ziehl- Neelson, Real-time PCR.

## INTRODUCTION

Intestinal Parasitic Infections are among the most common human infections worldwide, especially in poor societies and developing countries [Saneian *et al*., 2010], causing significant morbidity and mortality [Omorodion *et al*., 2012].

Cryptosporidiosis, the disease caused by *Cryptosporidium* which is zoonotic protozoan parasite of the Apicomplexa phylum and the Cryptosporidiidae family [Romero *et al*., 2001] and an important pathogen contributed significantly to diarrheal disease in both humans and

animals throughout the world [Fayer, 2004; Bouzid *et al*., 2013], is characterized by diarrhea, dehydration, and weight loss [ Santín *et al*., 2004]. *Cryptosporidium* can infect more than 170 species of vertebrates [Romero *et al*., 2001]. Infected individuals and animals shed the parasite in their feces in the form of oocysts as few as 5 days after initial infection and for approximately 5 weeks after the finish of diarrheal illness [Roberts & Janovy, 2000]. Humans acquire the parasite by ingesting it in its oocyst form after it is excreted in the stool of infected animals or people [Leav *et al*., 2003]. Though distributed worldwide and endemic in developing countries, Cryptosporidiosis is seen in developed countries in sporadic outbreaks mainly affecting children and people who are immunocompromised [Leav *et al*., 2003]. In developing countries, *Cryptosporidium* infections occur mostly in children younger than 5 years [Bern *et al*., 2000].

In *Cryptosporidium* affects epithelial cells of the small intestine and occasionally stomach, gall bladder, liver, trachea, and lungs in a number of mammals including humans [Hunter & Nichols, 2002].

In immunocompetent hosts, infections are generally restricted to the intestinal epithelium, causing an acute, self-limiting gastroenteritis [Downey, *et al*., 2008]**.** However, in AIDS patients and other immunocompromised individuals, infection can result in life-threatening, chronic diarrhea and may spread to extraintestinal locations [Ventura *et al*., 1997]. The initial reports of *Cryptosporidium* infection in mice were published by Tyzzer in 1907. In 1955, Slavin described the parasite as a potential cause of diarrhea in turkeys. Cryptosporidiosis in calves was subsequently recognized in the 1970s. But it was not until *Cryptosporidium* infections were reported as a cause of death in AIDS patients in the 1980s that the protozoan parasite became accepted as a significant zoonotic pathogen warranting. Control and treatment of Cryptosporidiosis is difficult and diagnosis of diarrhrea caused by cryptosporidiosis is not possible through routine stool examination unless specific request order by the physician. Detection and identification of *C. parvum* oocysts in fecal specimens is done by modified Zeihl-Neelson method and Real-time PCR test. The aim of present study was to determine the prevalence of *C. parvum* in patients at Wasit Province.

## MATERIALS AND METHODS

### Patients

This study was conducted in patients referred to Al Karamah Teaching Hospital and General Hospital of Martyr Fairuz in Wasit, Iraq during November 2015 to February 2016 was

assessed. A total of 100 fecal samples were collected from the recruited children of age from less than 1year to 11 years old with severe or persistent diarrhea. The samples were examined as soon as received.

**Modified Zeihl-Neelson**

This modified technique proved to be easy of being performed, rapid and highly practicable to detect oocysts of *Cryptosporidium* sp in faecal samples, Moreover, the technique allows the laboratory worker to investigate the presence of oocyst in several faecal samples concomitantly as well as the ready and clear identification of oocysts in the smears [Ortolani, 2000].

By using floatation technique before staining, 1 gm of fecal samples was concentrated [Ldzi & Esbroeck, 2010]. The oocyst of *C.parvum* was checked using modified Zeihl-Neelson method which is a sensitive and specific method to detect the parasite in stool [Hu, 2002]. To search for oocysts light microscope with oil immersion lens was used. These oocysts appear as pink to red, spherical to ovoid bodies on a blue or purple background. The control group were stool samples from age match children with no diarrhea in the period of past 72 hours were used.

The steps of Modified Ziehl-Neelsen method according to procedures described by Angus *et al*. (1981) and UK NEQAS (2015) are.

a) Faecal smears are made directly from the stool sample.
b) Allow to air dry.
c) Fix in methanol for 3 minutes.
d) Stain with strong carbol fuchsin for 15-20 minutes.
e) Rinse thoroughly in tap water.
f) Decolourise in acid alcohol (1% HCl in methanol) for 15-20 seconds.
g) Rinse thoroughly in tap water.
h) Counterstain with 0.4% methylene blue for 30-60 seconds.
i) Rinse thoroughly and air dry.
j) Examine using x40 and x100 objectives.

Oocysts of *C. parvum* do not concentrate well using standard concentration techniques and are identified using various staining techniques. Using the Modified Ziehl-Neelsen stain, the

oocysts are acid-fast. However, staining within a smear and between specimens can vary from unstained, to partial red staining, to complete staining.

In this technique, the oocysts appear as pink to red, spherical to ovoid bodies on a blue or purple background.

**Real-Time PCR master mix preparation**

Real-Time PCR master mix was prepared for specific primer by using AccuPower® DualStarTM qPCR PreMix kit [Bioneer, Korea], and done according to company instructions. These qPCR master mix reaction components were added into TaqM TaqMan probe qPCR master mix reaction. Then these are all placed in vortex tubes for mixing the components and centrifuge for 3000rpm for 3 minutes in Exispin centrifuge, after that transferred into MiniOpticon Real-Time PCR thermocycler.

**Real-Time PCR Thermocycler conditions**

Real-Time PCR thermocycler conditions was done according to primer annealing temperature and qPCR Syber green kit instructions.

**Real-Time PCR Data analysis**

qPCR data analysis was performed by calculating the threshold cycle number (CT value) that presented the positive amplification gene in Real-time cycle number.

**Data analysis procedures**

Data entry and analysis were done using SPSS version 16 computer software. The baseline characteristics of the study population were summarized using medians and ranges for continuous variables; simultaneously, proportions and frequencies were used for categorical variables. A p value less than 0.05 was considered as statistically significant.

## RESULTS AND DISCUSSION

One hundred children (age less than one year to 11 years old) suffered from watery diarrhea and abdominal pain who attended Al Karamah Teaching Hospital and General Hospital of Martyr Fairuz in Wassit, Iraq during November 2015 to February 2016 was examined.

Samples of feces were stained using modified Ziehl-Neelsen and examined under microscope for detection of *C. parvum*. Out of 100 samples 55 (55%) samples were positive for

*C.parvum*. As it is shown in table 1 the rate of *C.parvum* infection was the lowest in children with 29 (29%) positive samples and the highest with 71 (71%) negative sample.

Table 1 shows the comparison of modified Ziehl-Neelsen smears and Real-time PCR tests. The results of modified Zeihl-Neelson showed 29 (29%) samples positive for *Cryptosporidium* oocysts while Real-time PCR results revealed 26 (26%) samples positive. A seventy one samples were negative for *Cryptosporidium* oocysts using modified Ziehl-Neelsen smears but some generated positive results using the RT- PCR .

**Table 1: Comparison of Modified Ziehl-Neelsen and RT- PCR for diagnosis of *C. parvum*.**

| Result | No. of Samples | Modified Ziehl-Neelsen | RT-PCR |
|---|---|---|---|
| *C. parvum* positive | 29<br>29% | 29<br>29% | 26<br>26% |
| *C. parvum* negative | 71<br>71% | 0<br>0% | 7<br>7% |
| Total | 100<br>100% | 29<br>29% | 33<br>33% |

Modified Zeihl-Neelson of a fecal smear used as the gold standard for detection of *C.parvum* oocysts in stool. In clinical microbiology laboratories, this method is used to easily identify cryptosporidial oocysts. Although the concentration and staining procedures are timeconsuming and also required an experienced microscopists to check the slides, but it is affordable and allows at the same time to determine other parasites [Huang *et al*., 2004]. According to the results of the present study *C. parvum* showed an overall rate of 29 out of 100 (29 %) using modified Zeihl-Neelson method. The high rate of infection with *C. parvum* in Wassit area might be associated with contamination of drinking water [Abdulsadah *et al*., 2013].

The infection prevalence of *C. parvum* on average in the present study was similar to other studies in Iraq such as what was reported by Abdulsadah *et al*. (2013) whereas the prevalence of *Cryptosporidium* infection was significantly higher (59%) in children under one year old compared to children between (7-12)years old(4%), the study of Mallah and Jomah (2015) in which the total infection of *Cryptosporidium* examined by Ziehl-Neelsen was 21%, the results of Khalil (2000) in Mosul (20.52%), and the study of Ali (2008) in which the

prevalence of *C.parvum* infection was higher among children under one year old in Ramadi City [Ali, 2008].

The Real-time PCR test detects only intact *Cryptosporidium* oocysts, the rapid test detect antigen, which may persist after the patient stops shedding intact organisms. Therefore, the results of the current study is not false-positives but might represent recently cured cases but not in active disease like this study. The Real-time PCR test showed to be highly sensitive compared with the modified Ziehl-Neelsen stain for the detection of *C. parvum*. In high-prevalence populations, test such as the Real-time PCR test, with a high sensitivity should be used as screening test of diagnosis of the disease compared with the modified Ziehl-Neelsen stain.

There was no discrepancy between the present study and previous study in Iraq. The difference present in other studies resulted from varied techniques have been used in sampling, the varied environments where the studies have been conducted and varied methods used for determination of the disease.

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